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A simple pollen collection, dehydration, and long-term storage method for litchi (*Litchi chinensis* Sonn.)

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ABSTRACT

A simple method for pollen collection and storage was devised for three litchi cultivars, namely, 'Sanyuehong,' 'Shuidong,' and 'Guiwei'. This method could realize long-term pollen storage over periods of up to two years for artificial pollination and crossbreeding. The most suitable drying method for litchi pollen was the use of an air-blowing electric dryer at 35 °C for 6 h. The germination rate of pollen stored at different temperatures (30 °C, 25 °C, 15 °C, 4 °C, and −86 °C) was investigated. The pollen germination rate decreased to lower than 5% after 9, 16, 27, and 52 d at 30 °C, 25 °C, 15 °C, and 4 °C, respectively. A significant negative correlation was observed between pollen germination rate and storage time. For all litchi cultivars tested, pollen cryo-stored (−86 °C) for one year showed significantly higher germination rates than those stored under the other conditions. Pollen stored at −86 °C maintained a high germination rate of above 70% even after two years of storage. Thus, our results indicated that storage of pollen at 4 °C was suitable for field pollinations in the blooming season for up to two months. However, storage at −86 °C maintained the high germination rates required for artificial hybridization even in the second year of storage.

1. Introduction

Litchi (*Litchi chinensis* Sonn.), a member of the Sapindaceae family, is one of the most important subtropical fruits worldwide. Litchi has three types of functionally unisexual flowers, namely, the male, female, and pseudohermaphroditic flowers, which generally bloom in three waves, one female and two male waves (M_1 and M_2) (Joubert, 1986; Tindall, 1994). The transitions between the waves in the same inflorescence are distinct. Therefore, litchi is a typical out-pollinated plant, and fruit set can occur only if pollen from male flowers is transferred to the stigma of female flowers (Stern and Gazit, 1996). Insufficient pollination has been found to be one of the most important factors responsible for low yields in many field and orchard crops (Shivanna and Sawhney, 1997). Artificial pollination provides a solution to this problem. One method of artificial pollination is pollen supplementation, which requires the collection of sufficient amounts of pollen and the storage of viable pollen for short (hours to weeks) or long (months to years) periods. Therefore, studying the methods of pollen storage is necessary. Historically, litchi cultivar breeding has relied primarily on seedling selection. To date, more than 300 cultivars have been planted in the National Litchi Germplasm Repository located in Guangzhou, China, which was established in 1988 and is the largest litchi germplasm gene bank in the world (Sun et al., 2010). However, crop improvement in litchi through seedling selection is not sustainable because of long-term clonal and single-crop farming, which has led to the loss of many germplasm sources. Therefore, artificial crossbreeding has become the primary approach to develop new litchi cultivars. Storage of pollen is necessary for controlled pollination to achieve the desired breeding objectives and to solve some cultural constraints in fruit production. The pollination of early-flowering cultivars with pollen from late-flowering cultivars or the use of litchi pollen for distant hybridization with longan (*Dimocarpus longan* Lour.) or rambutan (*Nephelium lappaceum*) may require long-term storage of litchi pollen for up to one year. Pollen storage is essential not only for artificial pollination and breeding programs but also for germplasm conservation.

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Drying of anthers is an important step in pollen collection. Desiccation tolerance may differ among genotypes. Air-drying, desiccant-drying, or drying with absorptive materials can be used for small quantities of anthers (Craddock et al., 2000; Metz et al., 2000). However, these methods are not efficient and can be influenced by environmental conditions. Storage of pollen at low temperatures with good results has been attempted in a number of plant species, such as dogwood, protea, mango, and almond (Craddock et al., 2000; David van der Walt and Littlejohn, 1996; Dutta et al., 2013; Martínez-Gómez and Gradziel, 2002). Long-term storage of pollen is useful to conserve the gene pool and to overcome temporal and spatial isolation of the parent species in breeding programs. A method for pollen collection from freshly dehiscing anthers of litchi using cyclohexane has been reported by Chaudhury et al. (2010). In addition, cryopreservation (in liquid nitrogen) has been successfully applied to achieve long-term pollen storage over periods of up to four years. However, such methods require very sophisticated laboratory equipment. This study was conducted to develop a simple procedure for pollen collection, dehydration, and long-term storage of litchi.

2. Materials and methods

2.1. Plant material

The experiments were conducted in the Litchi Germplasm Repository, Guangzhou, China. The litchi cultivars 'Sanyuehong', 'Shuidong', and 'Guiwei' were used as test plants. The 25- to 30-year-old trees of these pollen parents were healthy and free from diseases and pests.

2.2. Anther collection

Male flower development of litchi was defined in three stages. In the first stage, the anther extended just beyond the receptacle, and the filaments were not observed. In the second stage, the filaments extended by half. In the third stage, the filaments extended farther before the anthers cracked. Fifty anthers were collected from each of the three stages for each cultivar. They were placed in 2 ml centrifugal tubes and then dried at 35 °C with an air-blowing electric dryer for 24 h until the anthers were completely dry. The tubes were vibrated to release the pollen from the anthers. About 2 ml of distilled water was added to the tubes. The centrifugal tube was placed in an ultrasonic oscillator for 15 min, and vortexed for 5 min to evenly suspend the pollen in water. Pollen quantity was calculated by the blood count method. A drop of the suspension was placed in a hemocyte counting plate (XB-K-25) to count the quantity of pollen under a microscope. The number of pollen in a large square (1.0 mm × 1.0 mm × 0.1 mm in size with 400 small squares) was counted. Each treatment was performed in triplicate. The quantity of pollen in one anther was computed as follows:

$$\text{The quantity of pollen/one anther} = (2 \times n/10^{-4})/50$$

where n represents the mean number of pollen in the large square, 2 represents 2 ml of suspension, 10^{-4} represents the volume of the large square, and 50 represents the number of anthers.

2.3. Pollen dehydration

The following methodology was used to determine whether dehydration affects pollen germination, and to develop a suitable dehydration method. An electric air-blowing dryer was used in the tests. The temperature in the dryer was set at 50 °C, 45 °C, 35 °C, and 30 °C to determine the effect of temperature on pollen dehydration and germination. Anthers (4 g) were spread uniformly in an open Petri dish with a diameter of 15 cm and then dried under the above temperatures. After drying for different drying times, the anthers were transferred to a 150 mesh stainless steel sieve with a fine brush to collect the pollen, and the pollen was placed in 2 ml cryovials.

2.4. Pollen germination

A small amount of pollen was placed at the bottom of a 2 ml centrifuge tube, to which 40 μL of distilled water was added. The tube wall was flicked to blend pollen and water. The centrifuge tube was placed horizontally in a constantly illuminated incubator at 28 °C for 2 h (Stern and Gazit, 1998). Pollen germination was observed under a microscope (40×). Twenty different fields of vision, each with at least 30 grains, were examined per treatment. Pollen grains were considered to have germinated when the pollen tube was at least as long as the diameter of the pollen grain.

2.5. Pollen storage

After desiccation, pollen grains were placed in 2 ml cryovials and stored at 30 °C, 25 °C, 15 °C, 4 °C, and −86 °C. A small amount of pollen was removed at regular intervals to estimate the germination rate. Pollen stored under −86 °C was thawed after storage by keeping the samples at room temperature for 5 min. After thawing, pollen was obtained to detect the germination rate using the aforementioned method.

2.6. Statistical analysis

Data were analyzed by ANOVA. The percentages of viable pollen that were below 30% or over 70% of the Arcsin P 0.5 transformation were used before ANOVA analysis. Statistical analyses were performed using SPSS 19.0 statistical software. Correlations were ranked as good, moderate, and poor when they had determination coefficients (R^2) higher than 0.64 ($R > 0.8$), in the range of 0.25–0.64 ($0.5 < R < 0.8$), and below 0.25 ($R < 0.5$), respectively. Graphs were generated using Excel 2010.

3. Results

3.1. Anther collection

Male litchi flower development consists of three developmental stages. The quantity of pollen in one anther was examined in each of the three stages. The maximum number of mature pollen grains was observed when the anther was in the third stage (the filaments extended farther before the anthers cracked). The increase in pollen number with the developmental stage of anther was similar among the cultivars (Table 1). Significant differences were observed among the three stages of flower development in the three cultivars. This indicated that third-stage anthers were the most suitable for pollen collection.

3.2. Pollen dehydration

The drying temperature had significant effect on pollen germination. The pollen germination rate was very low after drying at 50 °C in the three litchi cultivars; the maximum pollen germination rate was 12.2% for 'Sanyuehong', 5.55% for 'Shuidong', and 8.92% for 'Guiwei'. A negative correlation was observed between pollen germination rate and drying time at 50 °C (Fig. 1). However, the pollen germination rate increased slightly after drying at 45 °C, reaching maximum pollen germination rates of 14.75% for 'Sanyuehong', 7.71% for 'Shuidong', and 9.55% for 'Guiwei'. A significant negative